The Effect of Diapause on Stress Tolerance in Migratory Milkweed Bugs, *Oncopeltus fasciatus*

Research Thesis

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By

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Abstract

In response to short photoperiods in the fall, the large milkweed bug, *Oncopeltus fasciatus*, enters a reproductive diapause and migrates south, presumably to avoid adverse conditions and food shortages. Milkweed bugs are unique in that they are one of few temperate insects to migrate during diapause. Because milkweed bugs avoid adverse conditions by migrating, it is uncertain whether diapause increases environmental stress tolerance, as is typical of other types of insect diapause. In this study, we tested 1) whether diapause increases environmental stress tolerance, 2) whether food shortage during diapause further influences stress tolerance during diapause, and 3) whether heat shock proteins are upregulated during diapause to enhance stress tolerance. Milkweed bugs were reared under nondiapausing (25°C, 16:8 L:D) and diapausing (25°C, 8:16 L:D) conditions, and diapausing conditions with no food, to simulate seasonal changes in food availability. We found that both cold tolerance at -10°C and heat tolerance at 43°C were significantly higher in diapausing bugs relative to nondiapausing controls. However, restricting access to food had no further effect on thermal tolerance. To see whether heat shock proteins (hsps) were in part responsible for the increase in thermal tolerance, we measured expression of ten different hsp transcripts from five families. None of the transcripts were significantly upregulated in response to diapause; thus, we conclude that some other mechanism is responsible for increasing environmental stress tolerance during diapause.

Introduction

To cope with seasonal adverse conditions, many insects enter diapause, a programmed state of dormancy often characterized by metabolic suppression and enhanced stress tolerance (MacRae, 2010. Denlinger, 2002. Tauber & Tauber, 1976). Diapause, which occurs at a genetically determined life stage, is typically induced by photoperiod and confers enhanced tolerance to seasonal stressors such as temperature extremes, food shortage, and desiccating conditions (Denlinger, 2002). For example, increased thermal tolerance has been demonstrated in the egg diapause of *Bombyx mori* (Xu et al, 1995), the larval diapause of *Chymomyza costata* (Kostal et al, 2003), the pupal diapause of *Rhagoletis pomonella* (Lopez-Martinez & Denlinger, 2008), and the adult diapause of *Culex pipiens* (Rinehart et al, 2006), to name a few. This increase in stress tolerance is a critical adaptation for successful overwintering (Denlinger, 2002). In the spring, diapause is terminated in response to specific environmental cues, such as elevated temperatures or longer day-lengths (Tauber & Tauber, 1976. Denlinger, 2002). In many instances, enhanced stress tolerance during diapause is associated with upregulation of heat shock proteins (Rinehart et al, 2007), which function as chaperone proteins that maintain the structural integrity of cellular proteins in the face of extreme conditions (Feder & Hofmann, 1999).

The large milkweed bug, *Oncopeltus fasciatus,* undergoes a reproductive diapause in order to partake in a southward-bound migration (Dingle, 1974) following its food source, the milkweed plant (Genus *Asclepias*). The natural range of *O. fasciatus* extends from Canada to as far south as Argentina (Slater, 1964). Diapause is initiated by short photoperiods in the fall and is characterized by halted ovarian development and a reduced

proclivity towards mating (Dingle, 1974). Depending on weather conditions, migration may not begin until October or November (Dingle, 1972), putting migrants at risk of low temperature and other adverse conditions. While many studies have reported diapause-induced stress tolerance in sessile and non-migratory species (Denlinger, 1991), *O. fasciatus* provides a tractable model to investigate the link between stress tolerance and diapause in a migratory insect. This type of long-distance migration coupled with an overwintering period is rare in insects, with perhaps the only other conspicuous example in North America being the monarch butterfly, *Danaus plexippus* (Wassenaar and Hobson, 1998).

In this study, we compared the thermal tolerance of non-diapausing and diapausing *O. fasciatus*. Additionally, since food shortage contributes to the migratory phenotype and hormonal expression of diapause in this species (Rankin and Riddiford, 1977), we determined whether food availability impacts thermal tolerance during diapause. Lastly, we tested whether enhanced cold and heat tolerance during diapause correlated with expression of heat shock proteins.

Materials and Methods

Insects

Milkweed bugs were reared at 25°C on a diet of dry milkweed seeds and water. Bugs destined for direct, nondiapause development were held at 16:8 L:D photoperiod, while those programmed for diapause were transferred to 8:16 L:D as early-instar nymphs. To assess the effect of food availability on diapause-induced stress tolerance, a third group was reared under diapausing conditions but deprived of food following day 7 of adulthood, hereafter referred to as the Diapause No Food (N.F) group.

Measurement of stress tolerance

To measure thermal tolerance, groups of 10 adults were taken from each colony around day 14 of adulthood and placed into 50 mL vials plugged with cotton and sealed with parafilm. The bugs were exposed to temperatures of -6°C, -8°C, and -10°C for 2 hours in a programmable ethanol bath. After an overnight recovery period, survival was assessed. Bugs that could walk forward while holding their body above the substrate were considered alive. Heat tolerance was similarly measured at 40°C, 43°C, and 45°C for 2 h in a programmable bath containing 50:50 water:glycerol. At each temperature, we recorded survival for 4-8 independent replicates of 10 bugs each.

Gene expression analysis

Partial sequences for a single *hsp70* and *hsp90* transcript were obtained using PCR primers designed from conserved regions of insect heat shock protein sequences obtained from GenBank. The remaining eight sequences were obtained from a recently published transcriptome for *O. fasciatus* (Ewen-Campen et al, 2011). We designed qPCR primers for each transcript to amplify a 50-150 bp region of interest (Table 1). These primers were tested with PCR to ensure a single product of the expected size was produced. PCR products were then purified using the PureLink PCR Purification Kit (Life Technologies, Grand Island, NY) and sent to the Ohio State University Plant Microbe and Genomics Facility for Sanger sequencing to confirm the sequence of the amplicon.

Gene expression was analyzed in the Nondiapause, Diapause, and Diapause N.F. groups 14 d after adult emergence, as well as 28 d post emergence in the Diapause and Diapause N.F. groups. RNA was extracted using the Ambion Ribopure kit (Life

Technologies, Carlsbad, CA), according to the manufacturer's protocol. The quantity and purity of the RNA samples were evaluated using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). We then converted the RNA samples to cDNA using the Invitrogen Superscript III First Strand Synthesis System (Life Technologies, Grand Island, NY). For qPCR, each 20 μL reaction consisted of 2 μL cDNA template, 2 μL of each primer, 4 μL of molecular grade H_2O, and 10 μL of 2X Sybr Green Mastermix (Life Technologies, Carlsbad, CA). A Bio-Rad iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) was used to run the reactions. Each reaction began with 3 min at 94°C followed by 40 cycles of 10 s at 94°C, 30 s at the appropriate annealing temperature and 30 s at 72°C. Each reaction was followed by a melt-curve analysis to ensure that no other products were synthesized in the process. Gene expression was calculated using the $2^{-\Delta C_t}$ method, as in Teets et al. (2012). The C_t, or critical threshold value, of the reference gene, *rp49,* was subtracted from the C_t of the gene of interest to obtain the ΔC_t. The expression value for each sample was calculated using $2^{-\Delta C_t}$, which gives the relative expression of the gene of interest normalized to the control gene. The relative fold change for each treatment group was obtained by dividing the mean relative expression value ($2^{-\Delta C_t}$) of each treatment group by the mean expression value of the control group. To compare mRNA quantities between groups, ANOVA with a post-hoc Tukey test was conducted on the ΔC_t values using JMP 9 (SAS Institute Inc., Cary, NC).

Results

Confirmation of Diapause

To demonstrate that our lab reared colonies could successfully enter diapause, we collected oviposition data for individual pairs of milkweed bugs (Fig. 1). At 25°C, the nondiapausing females required an average of 10.1 d after emergence into adulthood to achieve first oviposition. Meanwhile, the diapausing individuals delayed their age of first oviposition to a mean of 22.7 d. When this experiment was repeated at a lower temperature of 23°C, the mean date of first oviposition was 25.4 d for nondiapausing individuals, while 6 of the 9 mating pairs reared under diapausing conditions failed to oviposit by 100 d, which is when the experiment was concluded. This illustrates that temperature plays a role in the expression of diapause.

Thermal Tolerance Assays

The mean survival of Nondiapausing bugs was between 95-100% following cold shock at -6 and -8°C and then dropped to 61.25% at -10°C. Meanwhile, the mean cold shock survival for Diapausing individuals was 97-100% for all three temperatures and was significantly higher at -10°C ($p<0.05$, ANOVA, Tukey; Fig. 2A). In the heat tolerance experiment, the mean survival in Nondiapausing bugs dropped from 100% at 40°C to 40% at 43°C, while the mean heat shock survival for diapausing individuals was 100% at 40°C and 72% at 43°C, with the difference in survival between diapausing and nondiapausing milkweed bugs at 43°C being statistically significant ($p<0.05$, ANOVA, Tukey) (Fig. 2B). Removing food from diapausing bugs did not have a significant impact on thermal tolerance (Fig. 2).

Heat Shock Protein Analysis

In several of the genes (*hsp70-1*, *hsp70-4*, *hsp90-1*, *hsp90-2*, *hsp27*, and *hsp60*), we failed to detect any significant differential expression. A slight, significant upregulation (~1.5-fold) was detected for *hsp70-2 (Fig. 3B)* in the Diapause N.F. day 14 group (ANOVA, Tukey, $p<0.05$). We also observed modest but significant downregulation of *hsp70-3 (Fig. 3C)* in the Diapause N.F. day 14, Diapause day 28, and Diapause N.F. day 28 samples. The transcript for *hsp23 (Fig. 3H)* was also found to be significantly downregulated in the Diapause day 28 sample. Finally, *hsp40 (Fig. 3J)* was downregulated in the Diapause day 28 group and the Diapause N.F. day 28 group.

Discussion

Overall, our results show that milkweed bugs enhance thermal tolerance during their migratory overwintering diapause. Survival following 2 h exposure to extreme cold and heat was significantly higher at -10°C and 43°C in diapausing adults. While seasonal disappearance of food source appears to be an important regulator of diapause characteristics such as juvenile hormone levels and migratory behavior (Ranking and Riddiford, 1977), food shortage did not enhance acute thermal tolerance of diapausing adults. Food shortage has been linked to enhanced cold tolerance in other arthropods. For example, in Collembola, entering a non-feeding state and ridding the gut of ice nucleating food particles is a crucial adaptation to increase winter cold hardiness (Worland and Convey, 2008. Somme and Block, 1982). Similarly, seasonal gut clearance has been observed in the beetle *Dendroides canadensis* to reduce the risk of freezing (Olsen and

Duman, 1997). However, since bugs were exposed only briefly to subzero temperatures well above the supercooling point (~-19°C for diapausing bugs; N.M. Teets, unpublished data), they were not at significant risk of freezing, which perhaps explains the failure of food shortage to improve cold tolerance. However, additional experiments using longer, ecologically relevant cold exposures, in which the risk of freezing would increase, are needed to fully determine whether food shortage influences cold tolerance in diapausing milkweed bugs. Furthermore, similar experiments could be done to examine cold shock effects on the diapausing and nondiapausing milkweed bugs' ability to produce viable offspring.

The augmented thermal stress tolerance in diapausing milkweed bugs is consistent with the diapause characteristics of nonmigratory insects. Another temperate Hemipteran that undergoes a photoperiodically controlled reproductive diapause, the firebug, *Pyrrhocoris apterus,* shows a similar enhancement of chill tolerance during diapause (Kostal and Simek, 2000). In many regards, the diapause characteristics of the milkweed bug are similar to those of monarch butterflies, perhaps the best-known model of seasonal migration in insects. Like the milkweed bug, monarch butterfly populations enter diapause in late-summer, in response to long-day conditions (Herman, 1981). Migratory individuals display delayed sexual development (Herman, 1981) and have enhanced cold shock tolerance as a component of diapause (Troyer at al, 1996). The overlaps between the diapauses of these two migratory insects suggest that analogous life histories and environmental components result in similar expressions of diapause. However, whereas monarchs are unamenable to laboratory rearing, milkweed bugs are abundant and easily

bred in captivity, thus making them ideal for studying the physiological intersection of diapause, migration, and stress tolerance.

A common mechanism permitting enhanced thermal tolerance during diapause is upregulation of heat shock proteins (Rinehart et al, 2007.Yocum, 2001. Goto et al, 1998). However, despite having higher cold and heat tolerance, there was no clear correlation between diapause status and increased abundance of heat shock protein transcripts in milkweed bugs. While we observed slight upregulation of *hsp70-1* in the Diapause N. F. group, this appears to be primarily a consequence of food shortage, as this transcript was not upregulated in diapausing individuals with access to food. Also, the observed fold-change (~1.5) was considerably lower than the upregulation typically found in diapausing insects; for example, *hsp70* is upregulated >6-fold during pupal diapause in the flesh fly, *Sarcophaga crassipalpis* (Ragland et al, 2010). Thus, it is difficult to predict what effects, if any, this slight upregulation would have. The observed downregulation of *hsp70-2* (Diapause N.F. 14, Diapause 28, and Diapause N.F. 28), *hsp23* (Diapause 28) and *hsp40* (Diapause and Diapause N.F. 28) most likely do not have implications on the milkweed bugs' ability to overcome extreme temperatures, since it would not be expected that downregulation of heat shock proteins would enhance survival. Our results were similar to those obtained in *C. pipiens*, which despite being more cold tolerant during diapause, does not upregulate the stress-inducible *hsp70* gene (Rinehart et al., 2006). Milkweed bugs do upregulate a thermal hysteresis protein during diapause (Patterson et al, 1981), which lowers the supercooling point of body fluids. However, since our test temperatures (-6 to -10°C) were well above the supercooling point, upregulation of thermal hysteresis proteins likely doesn't explain our results.

Future directions should consider the effect of blood sugars and polyols functioning as cryoprotectants. Accumulation of these metabolites, low molecular-weight solutes that reduce cellular damage by preventing cellular dehydration and stabilizing membrane lipids and proteins (Anchordoguy et al, 1987. Rudolph & Crowe, 1985), may be a key component for this species. Another strategy that warrants further attention is membrane lipid modifications. In order to prevent cellular membranes from solidifying at low temperatures, known as gel transition, changes may be made to the composition of the membrane such as increasing the proportion of unsaturated doubled bonds to increase fluidity (Kostal, 2010). Lastly, in some species, enhanced antioxidant defenses contribute to overwintering stress tolerance by minimizing the effects of cold-induced metabolic disruption (Joanisse & Storey, 1996; Ragland et al, 2010; Sim and Denlinger, 2011).

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Table 1 qPCR primers used to amplify heat shock proteins and the control gene, rp49

Transcript Name	Sequence	Annealing Temperature (°C)
hsp70-1	F: 5' – TTT TTG GTT TGG AGG AGT CG – 3'	53.1
	R: 5' – GCG AAG CGA CTA ATT GGA AG – 3'	54.0
hsp70-2	F: 5' – TCA TCA GGA TTG ATG GAC TTA TTA – 3'	51.3
	R: 5' – GAC AAA GCA CAG ATC CAT GAT A – 3'	52.7
hsp70-3	F: 5' – GGG GTT AAT TCC TCT AGA TGG TTC – 3'	54.7
	R: 5' – AGA AAG TCT TGG AGG ATG CTG ATA – 3'	55.5
hsp70-4	F: 5' – TCT TTG CCA GTT CCT TTG TCC CTG – 3'	59.5
	R: 5' – ATT GGT ATC CCA CCA GCA CCT AGA – 3'	59.5
hsp90-1	F: 5' – CCA CAC CAA ACT GAC CAA TCA T – 3'	60.8
	R: 5' – GGC ATT TAT GGA AGC TCT GC – 3'	60.4
hsp90-2	F: 5' – TGA AGA CAG TAC CAA CCG GAC CAA – 3'	60.0
	R: 5' – CCC TTC GCT TAA CCC TCT CAA CAA – 3'	59.0
hsp27	F: 5' – TGA AGT AGC CAC TCC TTG GTC GAA – 3'	59.8
	R: 5' – TGA ACG GCC TTC ACG TCT TCT TGA – 3'	60.5
hsp23	F: 5' – ACC AGA CGC TAC AAG ATC CCT GAA – 3'	59.7
	R: 5' – GCT TTC ACA GCT GGC TGT TGA GTT – 3'	60.2
hsp60	F: 5' – CAA GAG CCA TTG CCA AGG AAG GAT – 3'	59.7
	R: 5' – TGG TCT GTA ACA GCA TCA ACT GCC – 3'	59.4
hsp40	F: 5' – AAA CCT GGT TGG AGA GCA GGT ACT – 3'	60.0
	R: 5' – GGG TCA GGC TTG TCC CTT ATG ATG AA – 3'	60.4
rp49	F: 5' – AAG GGC CAG TAT TTG ATG CCC TCT – 3'	60.5
	R: 5' – CTA ACT GCG TGG GCA ATT TCA GCA – 3'	60.3

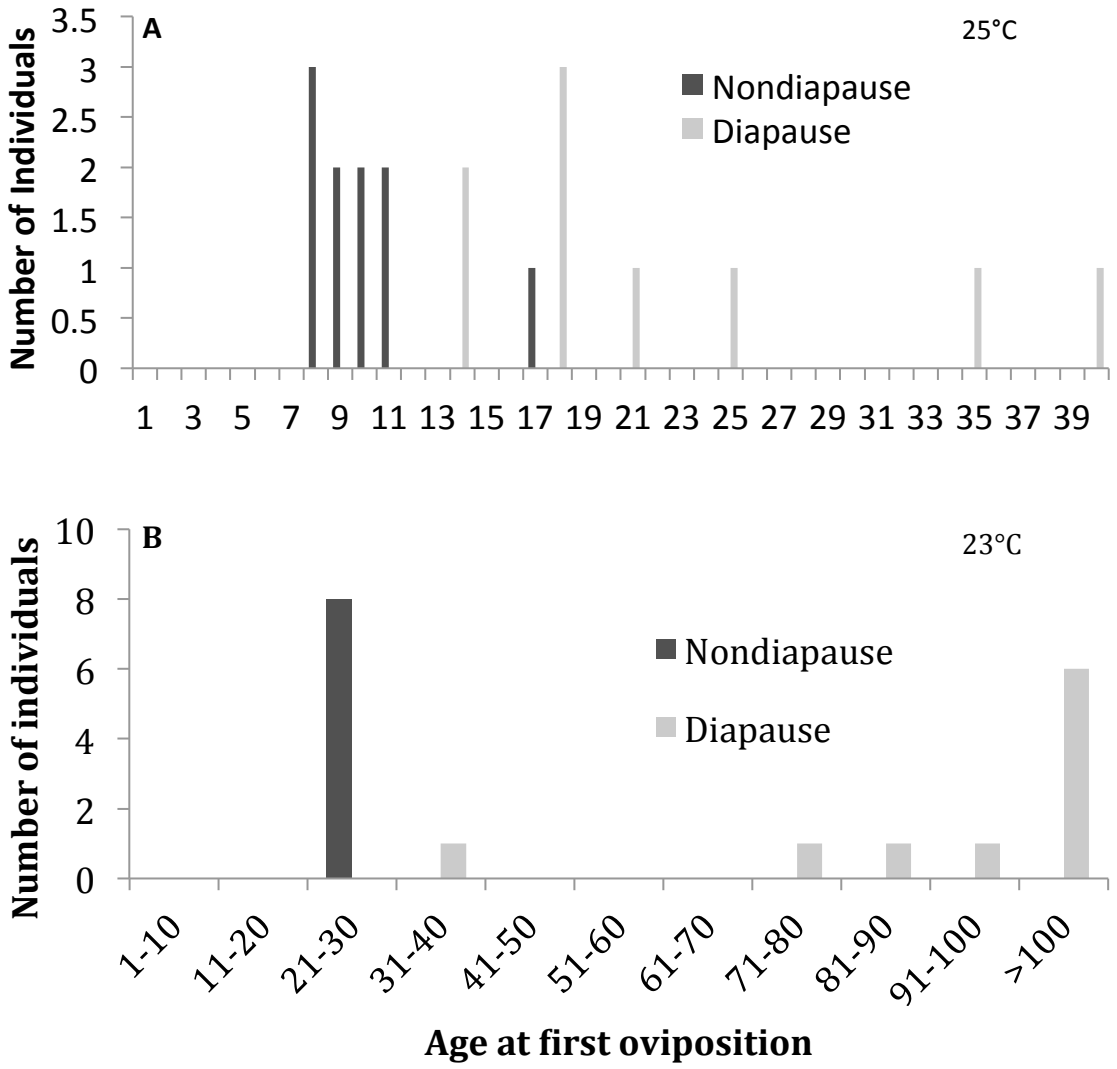

Fig. 1. Effect of diapause on the day of first oviposition. Data was collected by rearing pairs of male and female milkweed bug under Nondiapausng (16:8 L:D photoperiod) and Diapausing (8:16 L:D photoperiod) at 25 (A) and 23°C (B). Daily observations were made, and the day that each female began to oviposit was recorded. These results show that diapause affects the age at which females first oviposit and that lower temperature causes females to further delay oviposition.

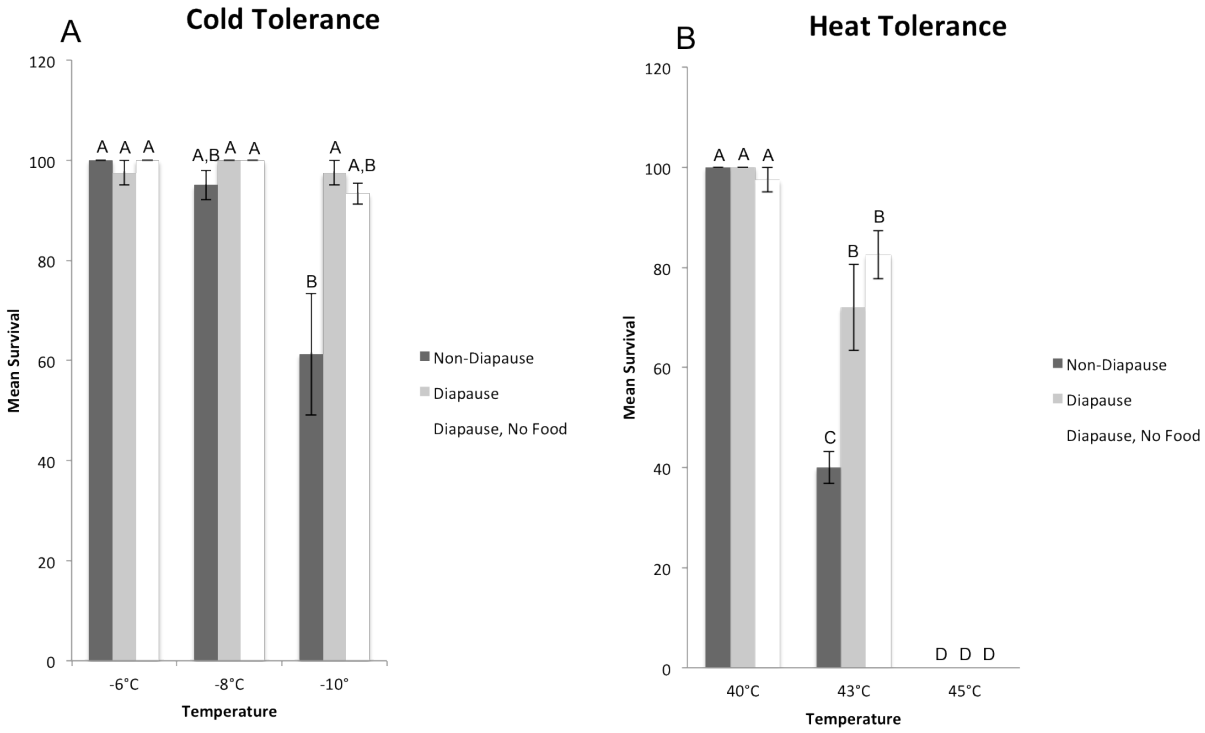

Fig. 2 Effect of diapause on (A) cold tolerance and (B) heat tolerance of adult milkweed bugs. Bars represent mean ± SE for each group. Different letters represent significant differences in milkweed bug survival.

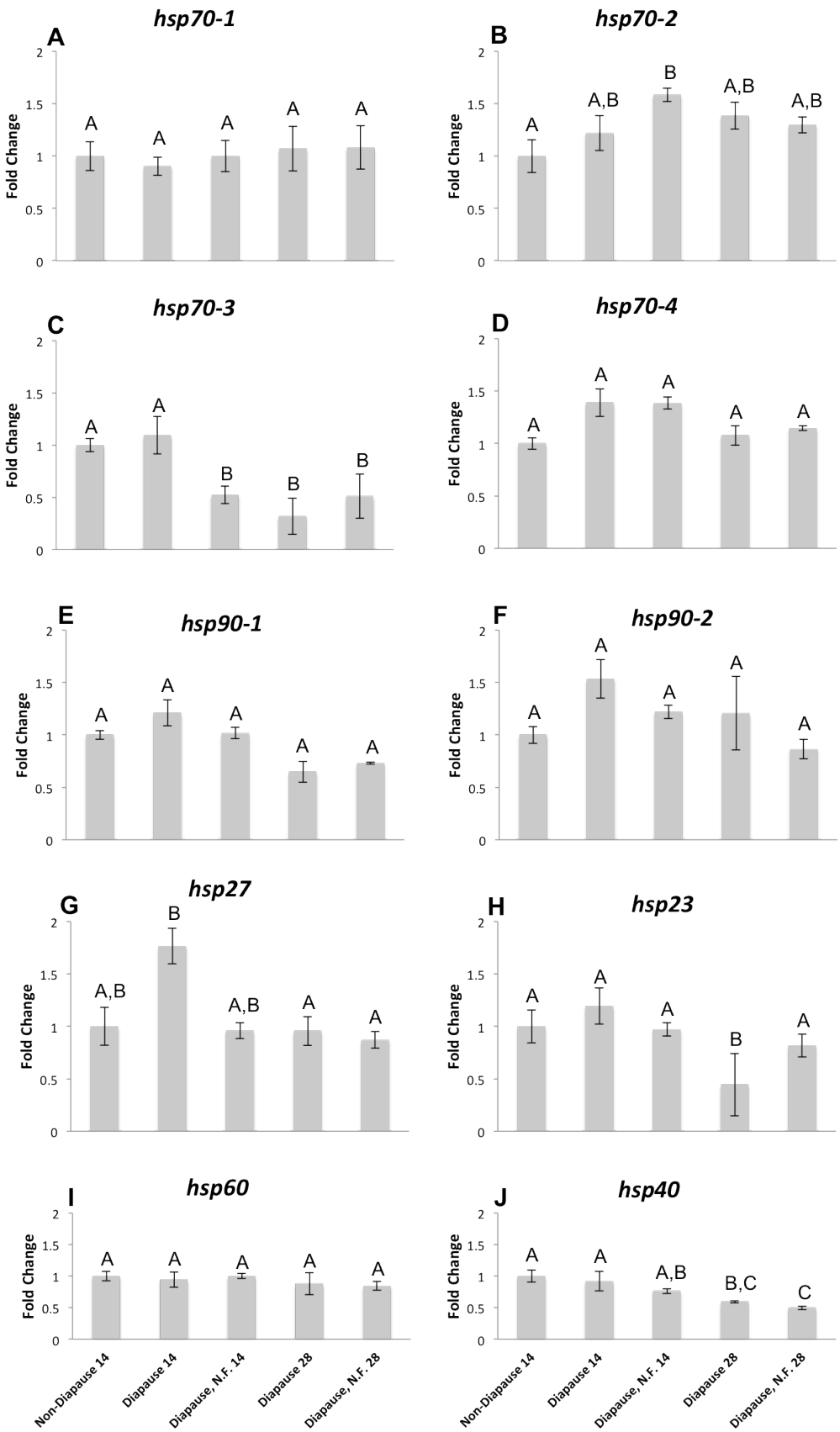

Fig. 3 Gene expression of the heat shock protein transcripts. The bars indicate mean SE fold change of each group ($p<0.05$, ANOVA, Tukey). Different letters represent significant fold differences in fold change.